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Im Auftrag

For the President of the European Patent Office

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Placental growth factor as a target for the treatment of osteoporosis

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Placental growth factor as a target for the treatment of osteoporosis

Field of the invention

This invention relates to antagonists of placental growth factor and signalling thereof, pharmaceutical compositions containing such antagonists and the use of such antagonists to prevent bone loss or bone mass and to enhance bone healing including the treatment of conditions which present with low bone mass and/or bone defects in vertebrates, and particularly mammals, including humans.

Background of the invention

Osteoporosis is a systemic skeletal disease, characterized by low bone mass and deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. In the U.S., the condition affects more than 25 million people and causes more than 1.3 million fractures each year, including 500,000 spine, 250,000 hip and 240,000 wrist fractures annually. Hip fractures are the most serious consequence of osteoporosis, with 5-20% of patients dying within one year, and over 50% of survivors being incapacitated. The elderly are at greatest risk of osteoporosis, and the problem is therefore predicted to increase significantly with the aging of the population. Worldwide fracture incidence is forecasted to increase three-fold over the next 60 years, and one study estimated that there will be 4.5 million hip fractures worldwide in 2050. Women are at greater risk of osteoporosis than men. Women experience a sharp acceleration of bone loss during the five years following menopause. Other factors that increase the risk include smoking, alcohol abuse, a sedentary lifestyle and low calcium intake. There are currently two main types of pharmaceutical therapy for the treatment of osteoporosis. The first is the use of anti-resorptive compounds to reduce the resorption of bone tissue. Estrogen is an example of an anti-resorptive agent. It is known that estrogen reduces fractures. In addition, Black, et al. in EP 0605193A1 report that estrogen, particularly when taken orally, lowers plasma levels of LDL and raises those of the beneficial high density lipoproteins (HDL's). However, estrogen failed to restore bone back to young adult levels in the established osteoporotic skeleton. Furthermore, long-term estrogen therapy, however, has been implicated in a variety of disorders, including an increase in the risk of uterine cancer, endometrial cancer and possibly breast cancer, causing many women to avoid this treatment. The significant undesirable effects associated with estrogen therapy

support the need to develop alternative therapies for osteoporosis that have the desirable effect on serum LDL but do not cause undesirable effects. A second type of pharmaceutical therapy for the treatment of osteoporosis is the use of anabolic agents to promote bone formation and increase bone mass. Although there are a variety of osteoporosis therapies there is a continuing need and a continuing search in this field of art for alternative osteoporosis therapies. In addition, there is a need for bone fracture healing therapies. Also, there is a need for therapy which can promote bone re-growth into skeletal areas where defects exist such as defects caused or produced by, for example, tumors in bone. Further, there is a need for a safer therapy with less side effects. In the art several studies have focussed on mechanisms of osteoclast activation. For example Niida et al (1999) have shown that vascular endothelial growth factor (VEGF) has a positive activity on osteoclast recruitment. One interesting homologue of VEGF is Placental growth factor (PlGF) but its role in bone has been poorly studied (Persico M.G. *et al*, 1999, Curr Top Microbiol Immunol 237, 31-40). US patent 5.919.899 describes PlGF and its use in the treatment of inflammatory disorders, wounds and ulcers. Several inhibitors for PlGF signalling, such as antibodies and tetrameric peptides, are known in the art and are disclosed in WO 01/85796. The present invention relates to the finding that antagonists of PlGF can be used for the manufacture of a medicament to suppress disorders of bone resorption such as osteoporosis.

Aims and detailed description of the invention

An object of the present invention is to provide a medicament for the treatment of osteoporosis in higher mammals exhibiting decreased cortical bone mineral density and preventing osteoporosis due to cortical bone mineral density reduction in such mammals. Another object of the invention is to provide pharmaceutical compositions useful in achieving the foregoing object. In our previous studies, the *PlGF* gene was inactivated in the mouse genome via homologous recombination in embryonic stem (ES) cells (Carmeliet P., 2000, J. Pathol. 190, 387-405, Carmeliet P., 1999, Curr. Interv. Cardiol. Reports 1, 322-335 and Carmeliet P. and Collen D., 1999, Curr. Top. Microbiol. Immunol. 237, 133-158). PlGF ($PlGF^{-/-}$) deficient mice are viable and fertile, and do not exhibit apparent bone defects. However, in the present invention it is shown that upon careful examination of bone histomorphometry, bone remodelling and biochemical analysis of these PlGF KO mice that PlGF plays an unexpected role in the

process of bone resorption. It is shown that PlGF deficiency results in decreased bone resorption, low bone turnover and increased trabecular bone mass. Thus the present invention shows that PlGF antagonists can be used for the manufacture of a medicament for treatment of bone disorders and more specifically for the treatment of conditions where there is an enhanced bone resorption such as for example osteoporosis.

Thus in one embodiment the invention provides the use of antagonists of PlGF for the manufacture of a medicament to treat bone resorption disorders. Antagonists of PlGF can suppress the bone resorption in said bone resorption disorders. In a specific embodiment said bone resorption disorder is osteoporosis. With "suppression" it is understood that suppression of bone resorption can occur for at least 20%, 30%, 30%, 50%, 60%, 70%, 80%, 90% or even 100%. More specifically the invention relates to the use of molecules (antagonists) to neutralize the activity of PlGF by interfering with its synthesis, translation, dimerisation, receptor-binding and/or receptor-binding-mediated signal transduction. By molecules it is meant peptides, tetrameric peptides, proteins, organic molecules, mutants of the VEGFR-1, soluble receptors of VEGFR-1 and any fragment or homologue thereof having the same neutralizing effect as stated above. Also, the invention is directed to anti-PlGF antibodies and functional fragments derived thereof, anti-sense RNA and DNA molecules and ribozymes that function to inhibit the translation of PlGF, all capable of interfering/or inhibiting the VEGFR-1 signal transduction. By synthesis it is meant trancription of PlGF. Small molecules can bind on the promoter region of PlGF and inhibit binding of a transcription factor or said molecules can bind said transcription factor and inhibit binding to the PlGF-promoter. By PlGF it is meant also its isoforms, which occur as a result of alternative splicing, and allelic variants thereof. As a result of alternative splicing, three PlGF RNAs encoding monomeric human PlGF-1, PlGF-2 and PlGF-3 isoform precursors containing 149, 179 and 219 amino acid residues, respectively, have been described. In normal mouse tissues, only one mouse PlGF mRNA encoding the equivalent of human PlGF-2 has been identified.

In a specific embodiment a murine monoclonal antibody against PlGF, disclosed in WO 01/85796 can be used for the manufacture of a medicament to treat bone resorption disorders. The term 'antibody' or 'antibodies' relates to an antibody characterized as being specifically directed against PlGF or its receptor (VEGFR-1) or any functional derivative thereof, with said antibodies being preferably monoclonal

antibodies; or an antigen-binding fragment thereof, of the $F(ab')_2$, F(ab) or single chain Fv type, or any type of recombinant antibody derived thereof. Preferably these antibodies, including specific polyclonal antisera prepared against PlGF or VEGFR-1 or any functional derivative thereof, have no cross-reactivity to others proteins. Monoclonal antibodies can for instance be produced by any hybridoma liable to be formed according to classical methods from splenic cells of an animal, particularly of a mouse or rat immunized against PlGF or VEGFR-1 or any functional derivative thereof, and of cells of a myeloma cell line, and to be selected by the ability of the hybridoma to produce the monoclonal antibodies recognizing PlGF or VEGFR-1 or any functional derivative thereof which have been initially used for the immunization of the animals. The monoclonal antibodies may be humanized versions of the mouse monoclonal antibodies made by means of recombinant DNA technology, departing from the mouse and/or human genomic DNA sequences coding for H and L chains or from cDNA clones coding for H and L chains. Alternatively monoclonal antibodies may be human monoclonal antibodies. Such human monoclonal antibodies are prepared, for instance, by means of human peripheral blood lymphocytes (PBL) repopulation of severe combined immune deficiency (SCID) mice as described in PCT/EP 99/03605 or by using transgenic non-human animals capable of producing human antibodies as described in US patent 5,545,806. Also fragments derived from these monoclonal antibodies such as Fab, $F(ab')_2$ and ssFv ("single chain variable fragment"), providing they have retained the original binding properties, form part of the present invention. Such fragments are commonly generated by, for instance, enzymatic digestion of the antibodies with papain, pepsin, or other proteases. It is well known to the person skilled in the art that monoclonal antibodies, or fragments thereof, can be modified for various uses. The antibodies can also be labeled by an appropriate label of the enzymatic, fluorescent, or radioactive type.

Small molecules, e.g. small organic molecules, and other drug candidates can be obtained, for example, from combinatorial and natural product libraries. To screen for said candidate/test molecules cell lines that express VEGFR-1 may be used and the signal transduction is monitored as described in detail in WO 01/85796 which is herein incorporated by reference. Said monitoring can be measured using standard biochemical techniques. Other responses such as activation or suppression of catalytic activity, phosphorylation (e.g. the tyrosine phosphorylation of the intracellular domain of the receptor) or dephosphorylation of other proteins, activation or modulation of

second messenger production, changes in cellular ion levels, association, dissociation or translocation of signalling molecules, or transcription or translation of specific genes may also be monitored. These assays may be performed using conventional techniques developed for these purposes in the course of screening. Inhibition of ligand binding to its cellular receptor may, via signal transduction pathways, affect a variety of cellular processes. Cellular processes under the control of the VEGFR-1/PlGF signalling pathway may include, but are not limited to, normal cellular functions, proliferation, differentiation, maintenance of cell shape, and adhesion, in addition to abnormal or potentially deleterious processes such as unregulated cell proliferation, loss of contact inhibition, blocking of differentiation or cell death. The qualitative or quantitative observation and measurement of any of the described cellular processes by techniques known in the art may be advantageously used as a means of scoring for signal transduction in the course of screening.

Random peptide libraries, such as tetrameric peptide libraries further described herein, consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to bind to the ligand binding site of a given receptor or other functional domains of a receptor such as kinase domains (Lam KS et al., 1991, Nature 354, 82). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that act to inhibit the biological activity of receptors through their interactions with the given receptor. Identification of molecules that are able to bind to the VEGFR-1 or PlGF may be accomplished by screening a peptide library with recombinant soluble VEGFR-1 protein or PlGF protein. For example, the kinase and extracellular ligand binding domains of VEGFR-1 may be separately expressed and used to screen peptide libraries. In addition to using soluble VEGFR-1 molecules, in another embodiment, it is possible to detect peptides that bind to cell surface receptors using intact cells. The cells used in this technique may be either alive or fixed cells. The cells will be incubated with the random peptide library and will bind certain peptides in the library to form a "rosette" between the target cells and the relevant solid phase support/peptide. The rosette can thereafter be isolated by differential centrifugation or removed physically under a dissecting microscope.

In another embodiment transdominant-negative mutant forms of VEGF-receptors (e.g. a transdominant-negative receptor of VEGF-R1) can be used to inhibit the signal

transduction of PlGF. The use of said transdominant-negative mutant forms of VEGF-receptors is fully described in US patent 5,851,999.

Also within the scope of the invention are oligoribonucleotide sequences, that include anti-sense RNA and DNA molecules and ribozymes that function to inhibit the translation of VEGFR-1 mRNA or PlGF mRNA. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the VEGFR-1 or PlGF nucleotide sequence, are preferred. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of VEGFR-1 or PlGF RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize anti-sense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

In a specific embodiment it should be clear that the therapeutic method of the present invention for the suppression of bone resorption can also be used in combination with any other therapy known in the art for the suppression of enhanced bone resorption.

The term 'medicament to treat' relates to a composition comprising molecules (antagonists) as described above and a pharmaceutically acceptable carrier or excipient (both terms can be used interchangeably) to treat diseases as indicated above. Suitable carriers or excipients known to the skilled man are saline, Ringer's solution, dextrose solution, Hank's solution, fixed oils, ethyl oleate, 5% dextrose in saline, substances that enhance isotonicity and chemical stability, buffers and preservatives. Other suitable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids and amino acid copolymers. The 'medicament' may be administered by any suitable method within the knowledge of the skilled man. The preferred route of administration is parenterally. In parental administration, the medicament of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with the pharmaceutically acceptable excipients as defined above. However, the dosage and mode of administration will depend on the individual. Generally, the medicament is administered so that the protein, polypeptide, peptide of the present invention is given at a dose between 1 µg/kg and 10 mg/kg, more preferably between 10 µg/kg and 5 mg/kg, most preferably between 0.1 and 2 mg/kg. Preferably, it is given as a bolus dose. Continuous infusion may also be used and includes continuous subcutaneous delivery via an osmotic minipump. If so, the medicament may be infused at a dose between 5 and 20 µg/kg/minute, more preferably between 7 and 15 µg/kg/minute.

In another embodiment antibodies or functional fragments thereof can be used for the manufacture of a medicament for the treatment of the above-mentioned disorders. Non-limiting examples are the commercially available goat polyclonal antibody from R&D Pharmaceuticals, Abingdon, UK or the chicken polyclonal antibody (Gassmann *et al.*, 1990, Faseb J. 4, 2528). Preferentially said antibodies are humanized (Rader *et al.*, 2000, J. Biol. Chem. 275, 13668) and more preferentially human antibodies are used as a medicament.

Another aspect of administration for treatment is the use of gene therapy to deliver the above mentioned anti-sense gene or functional parts of the *PlGF* gene or a ribozyme

directed against the PIGF mRNA or a functional part thereof. Gene therapy means the treatment by the delivery of therapeutic nucleic acids to patient's cells. This is extensively reviewed in Lever and Goodfellow 1995; Br. Med Bull.,51, 1-242; Culver 1995; Ledley, F.D. 1995. Hum. Gene Ther. 6, 1129. To achieve gene therapy there must be a method of delivering genes to the patient's cells and additional methods to ensure the effective production of any therapeutic genes. There are two general approaches to achieve gene delivery; these are non-viral delivery and virus-mediated gene delivery.

In another embodiment PIGF promoter polymorphisms can be used to identify individuals having a predisposition to acquire excessive bone resorption. Indeed, it can be expected that promoter polymorphisms can give rise to much higher or much lower levels of PIGF. Consequently, higher levels of PIGF can lead to a predisposition to acquire an excessive bone resorption disorder such as osteoporosis while much lower levels of PIGF can lead to a protection to acquire excessive bone resorption such as osteoporosis.

The following examples more fully illustrate preferred features of the invention, but are not intended to limit the invention in any way. All of the starting materials and reagents disclosed below are known to those skilled in the art, and are available commercially or can be prepared using well-known techniques.

Examples

1. Examination of the bone phenotype of PIGF knockout mice

PIGF deficient mice were described before in Carmeliet P et al. (2001) Nature Medicine. 7:575-583.

1.1. Bone histomorphometry

Bones were processed for bone histomorphometry as previously described (Daci et al, J Bone Miner Res. 2000, 15:1510-1516). Briefly, the bones were embedded undecalcified in methylmetacrylate and 4 µm thick longitudinal sections were cut with a rotary microtome (RM 2155, Leica, Heidelberg, Germany) equipped with a tungsten carbide 50° knife. Sections were stained according to Von Kossa to assess mineralized bone. The measurements were performed in a standardized area comprising most of the proximal tibial metaphysis, using a Kontron Image Analyzing

System (Kontron Electronic, KS 400 V 3.00, Eching bei Munchen, Germany). All parameters comply with the recommendations of the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (Parfitt et al, 1987).

An increase of 18% in trabecular bone volume was measured in the proximal tibial metaphysis of newborn PlGF deficient mice compared to WT mice. This increase became more pronounced (+ 42%; $p<0,05$) in 12 weeks-old PlGF deficient mice.

1.2. BMD and indices of bone remodeling

Trabecular bone mineral density (BMD) was measured in excised tibiae by peripheral quantitative computer tomography (pQCT) (XCT-960M; Nordland Medical Systems Inc.) as described (Dacio et al, cfr supra). Four cross-sections (one cortical at mid-diaphysis and three trabecular at the proximal epiphysis) were scanned, and the data were analysed using a threshold value of 200 mg/cm^3 to select for bone and to exclude soft tissue. Cortical and trabecular bones were separated by "concentric peel" with the inner core defined as trabecular bone.

Analysis by pQCT showed that the trabecular bone mineral density was increased in PlGF-deficient mice at 12 weeks (+ 30%; $p<0,05$), whereas cortical bone parameters were only minimally affected. These observations confirmed the histomorphometric data.

1.3. Biochemical analysis

Serum osteocalcin was measured by the in-house RIA described previously (Bouillon et al. 1992 Clin. Chem 38:2055-2060). Collagen cross-links were quantitated according to an assay previously described (Daci et al. cfr supra). Serum osteocalcin levels measured in PlGF-deficient mice of different ages were on average 30% lower compared to WT mice ($p<0,05$). Urinary excretion of collagen cross-links was reduced in 12 weeks-old knockout mice by 26% ($p<0,05$).

These data show that deficiency of PlGF in mice results in decreased bone resorption, low bone turnover and increased trabecular bone mass, showing an important role for PlGF in the process of bone resorption.

2. Mouse model for osteoporosis

An epidemiological correlation is suggested between osteoporosis and cardiovascular disease independent of age. The basis for this correlation is unknown. Atherosclerosis-susceptible mice receiving a high-fat diet develop osteoporosis as

reflected in a decrease in bone mineral content and bone mineral density (Parhami et al. J Bone Miner Res 2001, 16, 182-188). Apolipoprotein-E deficient (*ApoE*$^{-/-}$) mice were obtained from Dr. J. Breslow (The Rockefeller University, New York, USA). Mice had a mixed genetic background of 75% C57Bl/6 and 25% 129SvJ. Animals were weaned at 4 weeks of age and maintained on normal chow diet for 1 week, after which time they were fed the high fat/high cholesterol diet. For studying the role of PlGF antagonists *ApoE*$^{-/-}$ mice are intraperitoneally injected three times per week with PlGF antagonists.

Both male and female ApoE deficient mice on the high fat/high cholesterol diet showed a decrease in trabecular content by 37% ($p<0,05$) and 12% respectively and a decrease in trabecular density by 42% ($p<0,05$) and 15% respectively. The prevention of the decrease in both parameters is currently being studied in both female and male mice that receive PlGF antagonists.

3. Osteoclast formation

Osteoclast formation was studied using cocultures of primary osteoblasts and bone marrow cells, treated with 1,25-dihydroxyvitamin D_3. Briefly, the marrow cavity of the tibiae from 6- to 8- week-old mice was flushed with α-MEM, cells were collected by centrifugation and nucleated cells counted using Türk's solution. In coculture experiments, primary osteoblasts were plated at 2×10^4 cell/well in a 48-well culture plate and 24h later bone marrow cells were added at 10^5 nucleated cells/well. Primary osteoblasts derived from the PlGF knockout or WT mice were cocultured with the corresponding bone marrow cells. Cocultures were treated with 2×10^{-8} M 1,25-vitamin D_3 or vehicle on day 1, day 3 and stopped at day 6. At the end of the coculture period, adherent cells were rinsed with PBS, fixed with 4% formaldehyde in PBS for 10 min, treated with ethanol-acetone 50:50 (v/v) for 1 min, air-dried and stained for TRAP. Cells were incubated at room temperature in 0.1 M sodium acetate, pH 5.0 containing naphtol As-MX phosphate and fast red violet LB salt, in the presence of 10 mM sodium tartrate. The number or size of cells staining positively and containing 3 or more nuclei was determined.

The total number of osteoclasts formed in bone marrow-osteoblast cocultures of PlGF deficient mice was decreased with 10% ($p<0,05$) compared to WT cocultures. When counting only the largest osteoclasts, their number was 50% lower in PlGF deficient cocultures compared to WT cocultures.

The size of osteoclasts formed in WT bone marrow-osteoblast cocultures
without PlGF antagonists antibodies: 14260 μm^2
with anti-PlGF antibodies (Pl5D11F10 en Pl9F7D6, disclosed in WO 01/ 85796): 6150 μm^2 $p<0,001$

Thus it could be shown that the osteoclast formation and especially the maturation of osteoclast precursors to large multinucleated TRAP positive cells is affected by PlGF as deficiency or low levels of PlGF or addition of anti-PlGF antibodies resulted in decreased size of osteoclasts.

4. Bone resorption assay

Measurement of ^{45}Ca-release from cultured tibias was performed as previously described (Engsig et al; 2000 J Cell Biol 151, 87, 879-889). Briefly, on day 1, pregnant females (16 days post coitum) were subcutaneously injected with 100 µCi ^{45}Ca. Twenty-four hours later, tibias were isolated and cultured in media supplemented with ascorbate, glutamate and albumin. Right tibias were treated with MF1 (250 µg/ml/48 hours), left tibias served as controls. Media was changed every day and the amount of radioactivity released in the culture supernatant and remaining in the bones on day 4 of culture was determined.

It was shown that Ca-release in organ cultures of embryonic long bones was significantly decreased in PlGF deficient explants. The Ca-release is currently being measured by the addition of PlGF antagonists.

Claims

1. The use of antagonists of placental growth factor for the manufacture of a medicament to treat disorders of bone resorption.
2. The use according to claim 1 wherein said treatment of disorders of bone resorption is a suppression of bone resorption.
3. The use of antagonists according to claims 1 and 2 wherein said antagonists are selected from the group consisting of antibodies, peptides, tetrameric peptides, small molecules, anti-sense nucleic acids and ribozymes.
4. The use according to claims 1, 2 and 3 wherein said bone resorption is osteoporosis.
5. The use of placental growth factor promoter polymorphisms to identify individuals having a predisposition to acquire disorders of bone resorption.

Abstract

This invention relates to antagonists of placental growth factor and signalling thereof, pharmaceutical compositions containing such antagonists and the use of such antagonists to prevent bone loss or bone mass and to enhance bone healing including the treatment of conditions which present with low bone mass and/or bone defects in vertebrates, and particularly mammals, including humans.